DYNAMICS OF ECDYSONE SECRETION AND ACTION IN THE FLESHFLY SARCOPHAGA PEREGRINA [1]

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Ecdysone, the growth and molting hormone of insects, provokes a developmental response when it accumulates to "threshold titer" at certain "critical periods." Meanwhile, the titer of juvenile hormone dictates whether the developmental response will be molting or metamorphosis. These two generalizations epitomize the prevailing theory of ecdysone's action.

In support of the theory there is a wealth of evidence that the secretion of ecdysone is necessary for molting or metamorphosis. But whether the developmental response is triggered by a certain threshold titer of ecdysone is by no means assured. There remains the possibility that ecdysone is promptly and progressively utilized and inactivated after its release into the blood. Under that circumstance, the effects of the hormone might be cumulative within the target-organs without any marked change in its concentration in the blood or the insect as a whole.

Precisely this state-of-affairs appears to be the case when ecdysone provokes puparium formation of mature larvae of the fleshfly, *Sarcophaga peregrina*. In the present report we document the swift inactivation of ecdysone after its secretion into the blood. Moreover, we show that the overt developmental response, puparium formation, is triggered, not by the accumulation of ecdysone itself, but, rather, by a summation of the latent, covert effects of the hormone.

MATERIALS AND METHODS

1. *Experimental animals*

All experiments were carried out on larvae of *Sarcophaga peregrina*. This species was chosen for study because of its large size and the ease with which one can obtain unlimited numbers of carefully timed larvae. Thus, as documented by Ohtaki (1966) and diagrammed in Figure 1, the activation of the endocrine system is opposed or prevented as long as mature larvae are stored in contact with water. This inhibition is relieved when "wet larvae" are transferred to dry conditions. Therefore, the secretion of ecdysone can be timed from the "0-hour of dryness" until the initiation of puparium formation which, at 25° C, routinely takes place 16–17 hours later. Meanwhile, at any time during this period one

can obtain homogeneous groups of larvae by timing their exposure to dry conditions.

All the larvae used in the present study were reared in gallon glass jars containing pig liver. After three days of feeding at $24.5 \pm 1.5°$ C, the mature larvae emptied their crops and tried to crawl away from the food. For two further days they were stored in the same jars, to which water was added to maintain wet conditions. Then they were collected, washed in tap water, and placed in clean gallon jars containing sufficient water to cover the bottom. After two further days of storage at $24.5°$ C, the wet larvae were blotted with filter paper and either used immediately as "0-hour larvae" or aged in dry jars for two or more hours.

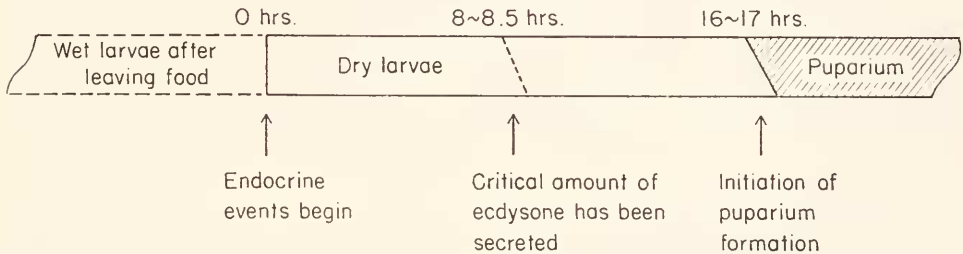FIGURE 1. The timing of developmental events when mature larvae of *Sarcophaga peregrina* are transferred from wet to dry conditions at 25° C.

2. *The Sarcophaga assay*

After exposure to dry conditions for 0, 2, 4, or 6 hours, homogeneous groups of larvae were ligated just behind the sixth segment. The majority of assays were performed on "standard test abdomens" which were prepared as follows:

Larvae were ligated after exposure to dry conditions for six hours. Twenty-four hours later, individuals showing puparium formation in front of, but not behind, the ligature were collected and aged an additional 16 hours under dry conditions. Larvae showing any trace of puparium formation behind the ligature were discarded; the number that did so ranged from 1 to 10%. The others were immediately used as "standard test abdomens."

Each assay was performed on a homogeneous group of 10 to 20 abdomens. The body anterior to the ligature was cut away and 10 μl of the test solution were injected by means of a 30-gauge glass or stainless steel needle sealed to a 100-μl microsyringe; the latter was controlled by an Agla micrometer. The needle was inserted through a loose ligature which was tightened immediately after the injection. When tests were performed on entire larvae, the injection was made through a ligature placed across the anterior tip just behind the mouthparts.

3. *The "puparium index"*

All assays were controlled 24 hours after injection. As recommended by Karlson (1956), each abdomen was scored as having undergone complete, marked, slight, or no puparium formation. These reactions were equated to 100, 75, 50, and 0%, respectively, for the purpose of calculating the "puparium index."

4. Ecdysone solutions

Synthetic α-ecdysone was obtained from Drs. John Fried and John Siddall of the Syntex Research Laboratory. The crystalline hormone was weighed, dissolved in 1 part warm isopropanol, and diluted with 9 parts water. Twofold serial dilutions were prepared in 10% isopropanol. One series of experiments made use of the phytoecdysones, β-ecdysone and ponasterone A, obtained through the courtesy of Profs. K. Nakanishi and T. Takemoto, of Tohoku University.

5. Collection and bioassay of larval blood

By means of iridectomy scissors a small sliver of integument was cut from the dorsal tips of the abdomens of a homogeneous group of ten washed larvae, care being taken not to damage the gut or other viscera. The larvae were immediately placed in a tilted Petri dish into which the blood was expressed by the spontaneous contractions of the cut larvae. The blood was immediately drawn into a 100-μl syringe and 10 μl injected into each of a series of test abdomens.

6. Extraction and concentration of ecdysone from the blood

The method was a modification of that described by Kaplanis *et al* (1966). Blood was collected from ten larvae, as just described. The volume was recorded and the blood immediately ejected into a conical centrifuge tube containing 4 ml of a 1:1 mixture of acetone and absolute ethanol. In this same manner additional blood was collected until a total of 1.5 ml had been obtained from a homogeneous group of approximately 100 individuals. The mixture was stirred at room temperature and then centrifuged at 1000 g for five minutes. The supernatant was decanted and the precipitate extracted twice more with 2-ml volumes of the solvent mixture. The supernatants were pooled and filtered with suction through a sintered medium-pore filter into a 50-ml round-bottom flask. The filter was rinsed with the solvent mixture and the extract reduced to dryness *in vacuo* on a rotary evaporator with temperatures rising to 50° C.

Crude extracts of this sort are extremely toxic when injected into larvae. Therefore, further purification was necessary. To this end, the contents of the flask were rinsed with 70% methanol into a small separatory funnel and washed with an equal volume of petroleum ether (*b.p.* 37–60° C). The methanolic hypophase was drained into a small round-bottom flask and reduced to dryness at 50° C as just described. By means of *n*-butanol the contents were rinsed into a separatory funnel and an equal volume of aqueous 2% sodium carbonate was added. The mixture was stirred, centrifuged, and the butanolic hypophase collected. The aqueous epiphase was twice re-extracted with 0.5 volume of butanol. The alcoholic phases were combined and washed twice with 0.5 volume of water. The aqueous phases were collected and re-extracted with 0.25 volume butanol and the latter washed with 0.5 volume of water. The butanolic phases were once again combined, placed in a round-bottom flask, and reduced to dryness on a rotary evaporator with temperatures rising to 65° C. With small volumes of methanol the contents of the flask were rinsed into a vial and the solvent evaporated in a stream of nitrogen. The extracted material was redissolved in 0.15 ml water, corresponding to one-tenth the original blood volume. This tenfold

concentrate was assayed by the injection of 10 μl into each of a series of isolated abdomens.

By the addition of known amounts of α-ecdysone the combined efficiencies of the extraction and purification procedures were found to be 90%. Therefore, in the present report, all extractions (except where otherwise noted) have been corrected for 10% loss.

7. Extraction and concentration of ecdysone from the entire animal

One to four larvae were homogenized in Ringer's solution and extracted with 4 ml of a 1:1 mixture of acetone and absolute ethanol. The mixture was centrifuged and the precipitate washed twice with a total of 4 ml of the solvent mixture. The supernatants were combined and the ecdysone extracted as described above for blood.

To harvest any ecdysone that might be complexed to proteins, the precipitate was reduced to dryness and treated with "Pronase." The reaction mixture consisted of 1 ml of 0.04 M Tris buffer (pH 7.5), 0.0066 M calcium chloride, and 1 ml of a 0.15% aqueous solution of the enzyme. After incubation at 40° C for 24 hours, the mixture was centrifuged and the supernatant decanted. The precipitate was twice washed with acetone-ethanol and the supernatants combined. The latter was purified and assayed as described above for blood. Additional extractions were also carried out on anterior and posterior ends of ligated larvae.

RESULTS

1. The effects of massive injections of ecdysone

As illustrated in Figure 1, 50% of larval abdomens undergo puparium formation when isolated after 8.5 hours of dryness. However, they require an additional 8 hours to do so. The significance of this latent period was examined in groups of 0-hour larvae injected with α-ecdysone, β-ecdysone, or ponasterone A. As summarized in Table I, even the largest doses failed to reduce the latent period to less than 8.5 hours.

2. Ecdysone secretion during exposure to dry conditions

Graded doses of α-ecdysone were injected into abdomens ligated after 0, 2, 4, and 6 hours of exposure to dry conditions. Each dose was tested in a homogeneous group of 20 abdomens. As shown in Figure 2A, the longer the preliminary exposure to dryness, the more sensitive were the abdomens to the injected ecdysone.

In Figure 2B the critical doses provoking a puparium index of 50% have been plotted as a function of the hours of exposure to dry conditions prior to ligation. These values define a *Sarcophaga* unit for abdomens isolated after 0, 2, 4, and 6 hours, respectively. Sensitivity to injected ecdysone increases at a rate that may be equated to about 0.007 μg per hour. It will be recalled that when ligation is postponed to the 8–8.5 hour, 50% of abdomens undergo puparium formation without any injection. Taken as a whole, these findings suggest that, at the outset of exposure to dry conditions, the endocrine system is activated and the ring-glands initiate the secretion of ecdysone at a fairly steady rate of about 0.01 μg per hour.

On the basis of Figures 2A and 2B one might suppose that abdomens isolated after 6 hours of dryness would be optimal for the biological assay. Unfortunately, this is not the case because up to 10% of the "6-hour abdomens" give false positive assays—*i.e.,* they undergo puparium formation without any injection.

TABLE I

Acceleration of puparium formation by injection of ecdysones into 0-hour larvae

Substance injected	Amount (μg)	Number injected	Time for 50% to initiate puparium formation (hrs)
Controls (solvent only)			
H_2O		20	17
10% Isopropanol		20	21
α-Ecdysone*	0.5	10	8.5
	1	10	8.5
	2	10	8.5
β-Ecdysone*	0.25	10	8.8
	0.5	10	9
	1	10	9
	2	10	9
Ponasterone A**	0.5	10	8.5
	0.7	10	8.5

* In 5 μl H_2O.
** In 5 μl 10% isopropanol.

Figure 2A also records the sensitivity of "standard test abdomens" which, as described above, are prepared by the further ageing of abdomens ligated after 6 hours of dryness. It will be observed that 95% of these abdomens have become less sensitive than at the time of their isolation. However, about 5% retain great sensitivity without showing any false positive assays. A puparium index of 50% is provoked by the injection of 0.035 μg α-ecdysone, which we define as a *"Sarcophaga* unit" for standard test abdomens (Ohtaki *et al,* 1967). This is the same average value that characterizes abdomens isolated and immediately used after 2 hours of dryness.

3. *Ecdysone assays of the blood*

On the basis of the foregoing results as well as Fraenkel's (1935) classic study of *Calliphora,* we anticipated that detectable amounts of ecdysone would be demonstrable in the blood. This prospect was tested by injecting standard test abdomens with blood obtained from donors at four successive stages—namely, larvae after 0, 6, and 12 hours of dryness; and 16-hour larvae showing the initiation of puparium formation. Each standard test abdomen received 10 μl of donor blood.

All assays were uniformly negative. So, by reference to Figure 2A, it appears that at no stage in puparium formation is as much as 0.0013 μg α-ecdysone present in 10 μl of blood. And since a mature larva contains 35 μl blood, the

total ecdysone in the entire blood could scarcely exceed about 0.005 μg, *i.e.,* 15% of a *Sarcophaga* unit for standard test abdomens.

4. *Extraction of blood ecdysone*

In order to bring this low titer within the range of the biological assay, we undertook the extraction and partial purification of blood ecdysone as described under Methods. The extract was redissolved in distilled water as a tenfold concentrate of the original blood, and 10 μl were injected into each of a series of standard test abdomens.
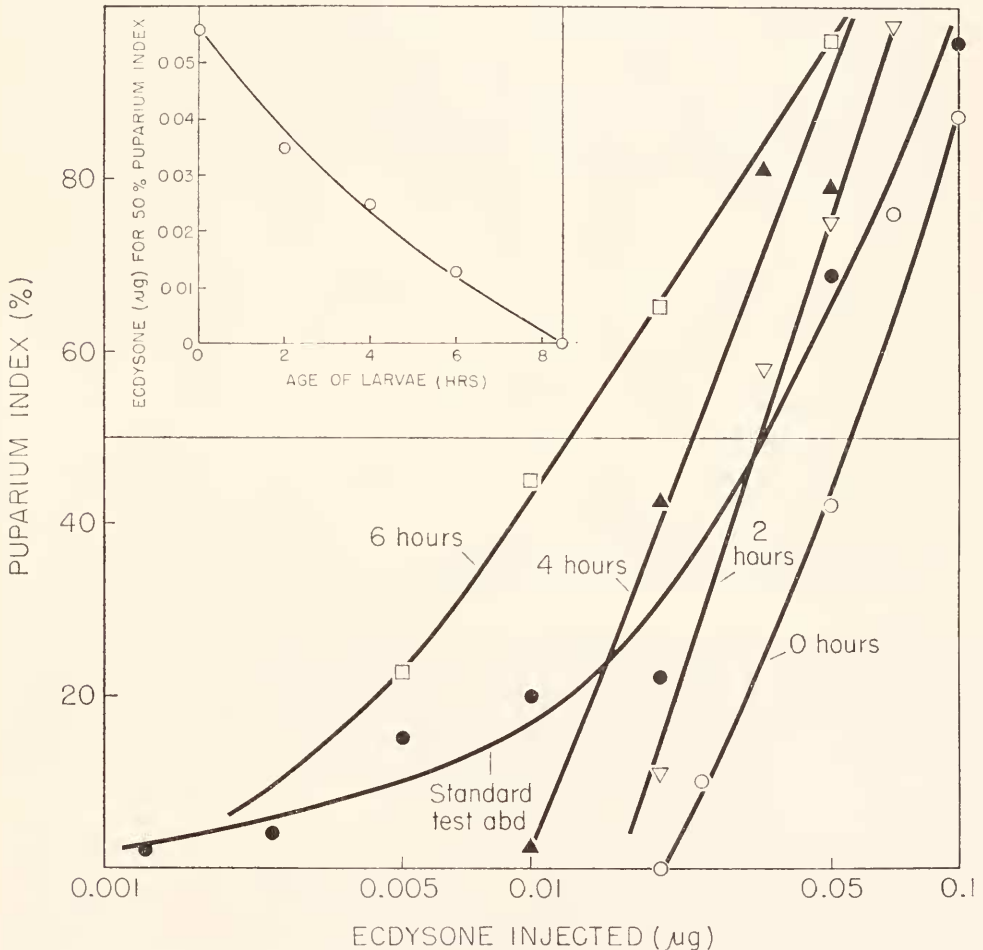

FIGURE 2A. The developmental responses (computed in terms of the puparium index) are plotted as a function of the amount of injected α-ecdysone. The curves correspond to abdomens ligatured after the indicated hours of exposure to dry conditions. For description of "standard test abdomens," see text.

FIGURE 2B. The inset in the upper left corner records the amount of injected ecdysone required for a 50% response of each type of abdomen.

Three concentrates of the blood of 0-hour larvae were assayed on a total of 41 standard test abdomens with the following result: 2 abdomens showed a trace of puparium formation; the other 39 were negative. This result corresponds to a puparium index of about 2%—a value which, as indicated in Figure 2A, is at the extreme limit of the calibration curve for standard test abdomens. Consequently, it appears that about 0.001 μg ecdysone is present in 10 μl of blood concentrate. On the assumption of 90% efficiency of the extraction procedure, we estimate that a total of 0.0004 μg ecdysone is present in the 35 μl of blood of a 0-hour larva, corresponding to about 1% of a *Sarcophaga* unit for standard test abdomens (see Table II).

The experiment was repeated on concentrates prepared from the blood of larvae at the 8th hour of exposure to dry conditions; 27 of 37 test abdomens showed no detectable reaction; 10 showed traces of puparium formation. This corresponds to a puparium index of 13% which, by reference to Figure 2A and on the assumption of 90% recovery, indicates the presence of about 0.0016 μg ecdysone in the entire blood of an 8-hour larva; *i.e.*, about 5% of a *Sarcophaga* unit (Table II).

TABLE II

Extraction of ecdysone from Sarcophaga peregrina

Stage	Ecdysone in:	
	Entire blood (μg)	Entire animal (μg)
0-hour larva	0.0004 (1%)*	0.0013 (4%)
8-hour larva	0.0016 (5%)	0.0025 (7%)
17-hour (white puparium)	0.005 (15%)	0.02 (57%)

* The bracketed figures record the corresponding percentage of a *Sarcophaga* unit as ascertained in assays performed on standard test abdomens.

In like manner the blood was collected from 100 individuals which had formed white puparia after 17 hours of exposure to dry conditions. Assays of the concentrated extract gave a puparium index of 34%, corresponding to about 0.005 μg α-ecdysone (15% of a *Sarcophaga* unit). This value is in accord with the upper limit of blood ecdysone calculated above in Section 3.

5. *Ecdysone in the entire larva*

The presence of so little ecdysone in the blood suggested that the hormone might be bound to the tissues. To test this possibility entire larvae were homogenized and extracted. The results summarized in Table II show that only about 4% of a *Sarcophaga* unit is present in an entire 0-hour larva, including the ring-gland itself. Even more surprising is the finding that only about 7% of a unit is present in an 8-hour larva, *i.e.*, at a stage where 50% of individuals have received all the ecdysone they require for puparium formation. The highest titer is found in white puparia where each individual contains about 57% of a *Sarcophaga* unit.

6. Recovery of injected ecdysone from the blood

Known amounts of α-ecdysone were injected into 0-hour larvae. Thereafter, at regular intervals, groups of injected larvae were sacrificed and their blood collected and assayed by injecting 5 μl into standard test abdomens. The puparium index was calculated in each case and equated to absolute units of α-ecdysone by reference to Figure 2A. The percentage of the injected ecdysone remaining in the blood was then calculated for each group.

The results summarized in Table III demonstrate that more than half of the injected hormone disappears from the blood within the first hour and that only traces remain after 8 hours.

TABLE III

Biological assays of blood* obtained from larvae injected with α-ecdysone

Ecdysone injected into blood donors (μg)	Time between injection and blood collection (hrs)	Number of test abdomens	Puparium index (%)	Equivalent ecdysone (μg)	% remaining in blood
2	1	10	100	>0.1	>35
	3	10	100	>0.1	>35
	5	20	62	0.047	16
	8	20	10	0.006	2
1	1	20	69	0.05	35
	3	20	23	0.015	11
	8	20	0	<0.001	<1
0.5	1	20	16	0.01	14
	3	20	8	0.004	6
	8	20	2	0.001	1

* Each standard test abdomen received 5 μl blood from donor larvae.

7. Recovery of injected ecdysone from entire larvae

One μg of α-ecdysone was injected into a series of 0-hour larvae. After exposure to room temperature for 0.1, 1, 3, or 8 hours, the entire larvae were homogenized and their ecdysone extracted and assayed in the usual way. Ninety per cent of the injected ecdysone was recovered from larvae extracted immediately after injection thereby testifying to the efficiency of the extraction and purification procedure. After correcting for this 10% loss, we found that one hour after the injection, only 50% was recovered; after three hours, 23%; and after eight hours, 2%.

The experiment was repeated except in this case the injected larvae were separated into blood and carcass. The blood was assayed by injecting 5 μl into standard test abdomens. The carcasses were cut open, rinsed in several changes of Ringer's solution, and then extracted and assayed in the usual manner.

The results summarized in Table IV once again document the rapid disappearance of injected ecdysone. And, here again, as in Table III, we note that the lower the dose, the more rapid is the relative rate of disappearance.

We entertained the possibility that ecdysone might be progressively bound to cellular proteins and in this manner rendered insoluble in the acetone-ethanol extraction mixture. To examine this possibility, 1 µg α-ecdysone was injected into each of ten 0-hour larvae. After three hours at room temperature, pairs of larvae were homogenized and five extracts prepared in acetone-ethanol. The precipitates were suspended in buffer and treated with "Pronase" as described under Methods. After incubation at 40° C for 24 hours, the reaction mixtures were once again extracted with acetone-ethanol to harvest any ecdysone that had been bound to proteins. These latter extracts were purified and assayed in the usual manner in a total of 54 standard test abdomens. All tests were uniformly negative.

TABLE IV

Recovery of injected α-ecdysone from blood and carcass of 1- to 8-hour larvae

Ecdysone injected (µg)	Time from injection (hr)	Number of animals	% of injected ecdysone recovered in		% not recovered
			Blood	Carcass*	
1.0	1	1	35	16	49
	3	2	11	13	76
	8	4	< 1	< 1	ca 99
0.5	1	1	14	9	77
	3	3	< 3	< 3	ca 95
	8	4	< 1	< 1	ca 99

* Corrected for 10% loss in the extraction procedure. Each experiment was performed in triplicate on the indicated number of animals. The average results are cited.

In control experiments, Pronase was found to have no deleterious effects on ecdysone. For example, two homogenates were prepared from uninjected larvae and 1 µg of α-ecdysone was added to each homogenate just prior to Pronase incubation. After 24 hours at 40° C, the reaction mixture was extracted with acetone-ethanol and purified in the usual way. The biological assays indicated the recovery of 100% of the added ecdysone.

For these several reasons the swift disappearance of injected ecdysone is accountable only in terms of its equally swift inactivation within the living insect.

8. *Stability of injected ecdysone at low temperature*

Three 0-hour larvae were placed at 2° C for 24 hours and then injected with 1 µg α-ecdysone. After an additional 24 hours at the low temperature, the larvae were homogenized and extracted. Biological assays of the extract showed the recovery of 100% of the injected hormone. Clearly, the inactivation of ecdysone is blocked by low temperature.

9. *Stability of injected ecdysone in the absence of oxygen*

Nine 0-hour larvae were placed in a covered Buchner funnel and exposed to a stream of nitrogen for 1 hour. Each individual was then injected with 1 µg

α-ecdysone. After three additional hours in nitrogen, the entire larvae were homogenized and extracted. Assays showed the recovery of 90% of the injected ecdysone. Control experiments performed on entire larvae exposed to air showed the recovery of only 20% of the injected ecdysone. In additional control experiments, 0-hour larvae underwent full and complete recovery after 4 hours of exposure to nitrogen.

10. *Inactivation of ecdysone by larval fragments*

The gut was removed from four 0-hour larvae. These larvae were then cut into small fragments and placed in 2 ml Ringer's solution containing 1 mg streptomycin sulfate. The mixture was subdivided between two small flasks, one of which contained 3 mg of the potent anti-tyrosinase, phenylthiourea (PTU). One μg of α-ecdysone was added to each flask and the latter incubated at 25° C on a gyratory table. After 24 hours the ecdysone was extracted and assayed. The recovery was 40% in the mixture containing PTU and 22% in the mixture not containing PTU.

The experiment was repeated using a shorter incubation time and cut-up larvae from which the gut was not removed. After 8 hours, 94% of the added ecdysone was recovered from the mixture containing PTU and 70% from the mixture not containing PTU.

11. *Experiments on homogenates and subcellular fractions*

When the just-mentioned experiment was repeated using crude homogenates prepared from 0-hour larvae, 100% of the added ecdysone was recovered from all the reaction mixtures after 24 hours of incubation. The same was true when ecdysone was incubated with blood collected from 0-hour larvae.

On the assumption that ecdysone inactivation might be blocked by the presence of one or more endogenous inhibitors, the crude homogenate was fractionated prior to testing. To this end, two 0-hour larvae were cut into fragments and rinsed for 2 minutes in 2 ml 0.01 M HEK buffer (pH 7.4) made up in 20% sucrose (Kafatos, 1968, page 1258). The fragments were collected and homogenized in 2 ml of sucrose-free HEK buffer. The homogenate was centrifuged at 1000 g for 3 minutes. The supernatant was collected and centrifuged for 50 minutes at 200,000 g, and the particulate fraction was combined with the low-speed sediment. The insoluble fraction was diluted with 2 ml Ringer's solution. One mg streptomycin and 1 μg α-ecdysone were added to each reaction mixture and the latter incubated at 25° C on a gyratory table. Eight hours later, the ecdysone was extracted and assayed. Recovery of added hormone was 100% for both reaction mixtures. The experiment was twice repeated with the same result. Thus, as in the case of crude homogenates, ecdysone inactivation was not brought about by any of the subcellular fractions. This same negative result was observed in additional experiments in which 1 mg/ml of NAD or NADH was added to the reaction mixture prior to incubation.

Discussion

1. Ecdysone titer in relation to puparium formation

At the outset of exposure to dry conditions a 0-hour larva contains 1.3 nanograms of ecdysone, most of which may be sequestered in its ring-gland (Table II). Fifty per cent of abdomens isolated at this time undergo puparium formation when injected with 55 nanograms of ecdysone (Fig. 2). Since the abdomen makes up 55% of larval mass, we calculate that an entire larva deprived of its ring-gland would require the injection of approximately 100 nanograms. When the ligation is postponed until the 8th hour of dryness, the ring-gland has already secreted sufficient ecdysone to cause 50% of abdomens to form puparia without any injection. Despite this fact, the amount of ecdysone in an 8-hour larva is only 2.5 nanograms (Table II). No less than 97 nanograms of ecdysone seem to have disappeared.

This same paradox is seen in the extractions and assays which Shaaya and Karlson (1965) and Shaaya (1967) have carried out on entire *Calliphora* larvae. From their data we calculate that at no stage prior to purparium formation does the titer of ecdysone exceed 26% of a *Calliphora* unit.

2. The half-life of injected ecdysone

All our experiments direct attention to the lability of α-ecdysone after its injection or its secretion by the ring-gland. Thus, as we have seen, within 1 hour after the injection of 1 μg, 50% had been inactivated; and after 8 hours, about 98% had been inactivated. The rate of inactivation was even higher in larvae injected with 0.5 μg. Therefore, for mature larvae of *Sarcophaga peregrina* at 25° C, we can state that the time for half-inactivation of α-ecdysone is less than 1 hour.

3. The inactivating mechanism

As described in Section 11 of Results, no trace of inactivation could be detected when α-ecdysone was incubated for 24 hours with larval blood or with crude homogenates prepared from 0-hour larvae. The same negative results were observed in tests performed on the soluble and the particulate fractions. By contrast, clear-cut inactivation occurred when α-ecdysone was incubated with larval fragments; *i.e.*, under conditions where the integrity of the cells was largely preserved. This demonstrates that the inactivating mechanism is present in at least some and perhaps most cells. As mentioned in Section 10 of Results, inactivation was brought about by larval fragments, irrespective of whether the gut was present or absent. Moreover, in additional experiments not mentioned above, inactivation of injected ecdysone took place in both anterior and posterior parts of ligated larvae. Inactivation by the anterior end was substantially faster than by the posterior end.

Our inability to demonstrate the inactivating mechanism in crude or purified homogenates implies the lability of the system or the requirement of co-factors and incubation conditions that were not satisfied. In Sections 8 and 9 of Results we observed that inactivation is blocked in larvae exposed to low temperature or to anaerobic conditions. Therefore, chemical and, more particularly, oxidative processes appear to be principally concerned.

4. *The dynamics of ecdysone action*

The rapid inactivation of ecdysone, demonstrated for the first time in the present study, directs attention to the accumulation, not of the hormone itself, but of the covert effects of the hormone. This new interpretation is summarized in the flow-sheet diagrammed in Figure 3. Ecdysone is secreted into the blood, taken up by the tissues, and reacts with the hormonal receptors to exert its primary action, whatever that may be. As a result of this primary reaction, a concatenation of biochemical and biophysical events is set in motion which comprise the covert effects of the hormone. The latter undergo, as it were, spatial and temporal summation within the target organs and finally trigger the overt effects, *i.e.*, the initiation of molting or metamorphosis. Meanwhile it may be noted that the covert
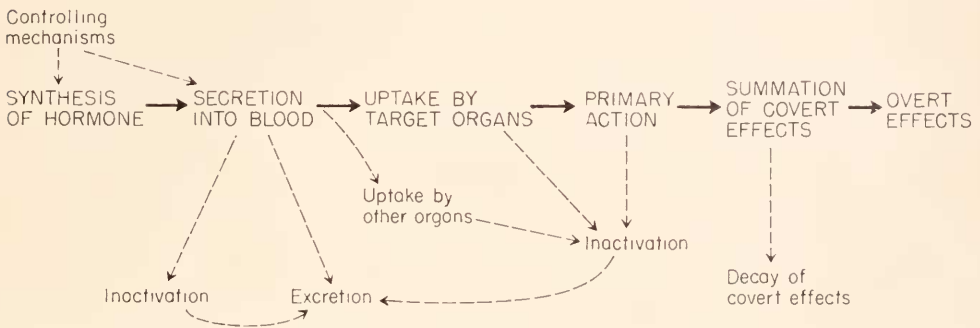

FIGURE 3. A revised theory of the hormonal action emphasizing the accumulation of the covert effects of ecdysone.

effects are subject to decay if their temporal accumulation is interrupted short of the discharge level. This fact is documented in Figure 2A in terms of the lessened sensitivity to ecdysone injection of standard-test abdomens as compared to 6-hour abdomens from which they were derived. Moreover, the accumulation and implementation of the covert effects require a certain finite time. Thus, as shown in Table I, even the largest doses of α-ecdysone, β-ecdysone, or ponasterone A required at least 8.5 hours to provoke the initiation of puparium formation. This minimal "latent period" apparently corresponds to the time required for the covert effects of ecdysone to accumulate to the discharge level.

It has not escaped our attention that this conception of the dynamics of ecdysone action may be applicable to hormones in general.

5. *Transfusion versus parabiosis*

The literature pertaining to insect endocrinology is replete with carefully controlled experiments in which hormonal effects have been conveyed between donors and recipients by joining them in parabiosis. By contrast, there are few claims to accomplishing this same goal by the injection or transfusion of blood. One of these rare claims ranks among the most famous experiments in the history of insect physiology; namely, Fraenkel's (1935) induction of puparium formation by the injection of "pupation blood."

During the past 33 years the literature is silent in confirming or refuting this historic experiment. Now, for reasons set forth in the present study, it appears that Fraenkel's results were "false positives" such as were described above in Section 2 of Results. This seems all the more likely since Fraenkel's experiments were not controlled by the injection of any materials other than "pupation blood." Needless to say, our very ability to resolve this paradox owes a great deal to Fraenkel's insightful pioneering work.

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Summary

1. Ecdysone is in a highly dynamic state after its injection or its secretion by the ring-gland of *Sarcophaga peregrina*. Hormonal activity is rapidly destroyed by an inactivating mechanism which is present in the tissues but not in the blood.

2. Inactivation is blocked by low temperatures or anaerobic conditions—a finding that implicates chemical and, more particularly, oxidative reactions. The mechanism in question could be demonstrated in larval fragments but not in crude or fractionated homogenates.

3. When injected into mature larvae, 1 μg of α-ecdysone loses 50% of its activity in 1 hour and 98% in 8 hours. Lower doses show even briefer "half-lives."

4. The rapid inactivation of ecdysone can account for its low titer in both the blood and tissues. Thus at the "critical period" for puparium formation, the entire larva contains only 2.5 nanograms, corresponding to only 7% of a *Sarcophaga* unit.

5. The evidence points to the accumulation, not of the hormone itself, but the covert biochemical and biophysical effects of the hormone. The covert effects undergo spatial and temporal summation within the target organs and finally discharge the overt developmental response.

6. The role of the blood is to serve, not as a reservoir, but as a pipeline through which ecdysone flows from the ring-gland to its sites of action and swift inactivation.

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